IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE APPLICANT	: Jackowski *et al.*
INVENTION	: **Fibronectin Biopolymer Markers Indicative of Type II Diabetes**
SERIAL NUMBER	: 09/991,796
FILING DATE	: November 23, 2001
EXAMINER	: Chernyshev, Olga N
GROUP ART UNIT	: 1649
OUR FILE NO.	: 2132.109

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CERTIFICATE UNDER 37 CFR 1.8(a)
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DECLARATION UNDER 37 CFR § 1.132

I, Ferris H. Lander, do hereby declare as follows:

1. I am a registered Patent Agent and am authorized to represent the inventor's and assignee in the application entitled **"Fibronectin Biopolymer Markers Indicative of Type II Diabetes"**, having U.S. Application Serial No. 09/991,796, filed November 23, 2001.

2. In the Office Action mailed on November 4, 2005, claim 1, (as presented on May 16, 2005) was rejected under 35 USC 101 because the claimed invention allegedly has no apparent or

disclosed specific and substantial credible utility. Claim 1 was also rejected under 35 USC 112, first paragraph since the claimed invention is not supported by either a clear asserted utility or a well established utility, according to Examiner, one skilled in the art clearly would not know how to use the claimed invention.

Specifically, the Examiner asserts that the specification does not support Applicants' statement that the presence of the claimed biopolymer markers (SEQ ID NOS:1 and 4) in a sample is indicative of a link to Type II diabetes. The Examiner maintains that the claimed biopolymer markers (SEQ ID NOS:1 and 4) are only useful for further research.

3. Applicants submit that Figures 1-4, as originally filed, are "evidence of record" which supports Applicants' possession of the claimed peptides (SEQ ID NOS:1 and 4) and their relationship to Type II diabetes.

(a) Figure 2 (as originally filed) shows a mass spectral profile obtained from Band 1 of the gel shown in Figure 1. Expression of the claimed peptide (SEQ ID NO:1) was shown, in Figure 1, to be present in serum samples obtained from normal patients and not present in serum samples obtained from Type II diabetes patients. Thus, the claimed peptide (SEQ ID NO:1) is differentially expressed in Type II diabetes versus normal.

(b) Figure 4 (as originally filed) shows a mass spectral profile obtained from Band 1 of the gel shown in Figure 3. Expression of the claimed peptide (SEQ ID NO:4) was shown, in Figure 3, to be present in serum samples obtained from normal

patients and not present in serum samples obtained from Type II diabetes patients. Thus, the claimed peptide (SEQ ID NO:4) is differentially expressed in Type II diabetes versus normal.

4. (a) In order to further illustrate this point, Applicants provide the attached figure entitled "DEAE 1(Elution) Normal vs. Diabetes Type II" which represents Figure 1 as originally filed. The attached figure was produced by scanning the original photograph of the gel. Expression of Band #1 is shown in samples obtained from patients determined to be normal with regard to Type II diabetes (lanes 1-4, as read from the left) and is not shown in samples obtained from Type II diabetes (lanes 5-9). Thus, the claimed peptide (SEQ ID NO:1) is shown to be differentially expressed between Type II diabetes and normal controls. No new matter has been added; this figure is simply a clearer copy of Figure 1 as originally filed and is provided to clarify the presence and differential expression of the claimed biopolymer marker (SEQ ID NO:1). The gel shown in the figure does not represent new experimentation; the figure shows a clearer image of the original gel made at the time that the experiments described in the instant specification were first carried out.

(b) In order to further illustrate this point, Applicants also provide the attached figure entitled "HiQ3 (scrub) Normal vs. Diabetes Type II" which represents Figure 3 as originally filed. The attached figure was produced by scanning the original photograph of the gel. Expression of Band #1 is shown in samples obtained from patients determined to be normal with regard to Type

II diabetes (lanes 7-10, as read from the left) and is not shown in samples obtained from Type II diabetes (lanes 2-6). Thus, the claimed peptide (SEQ ID NO:4) is shown to be differentially expressed between Type II diabetes and normal controls. No new matter has been added; this figure is simply a clearer copy of Figure 3 as originally filed and is provided to clarify the presence and differential expression of the claimed biopolymer marker (SEQ ID NO:4). The gel shown in the figure does not represent new experimentation; the figure shows a clearer image of the original gel made at the time that the experiments described in the instant specification were first carried out.

The undersigned declares that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the Application or any patent issuing thereon.

2/6/2006.
Date

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Reg. No. 43,377

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Examiner T09 reference 7

Fibronectin Fragments Modulate Human Retinal Capillary Cell Proliferation and Migration

2132.109 Examiner copy reference #1

Maria B. Grant, Sergio Caballero, David M. Bush, and Polyxenie E. Spoerri

Capillary morphogenesis involves cell-cell and cell-matrix interactions. Proteases elaborated by capillary cells modify the extracellular matrix (ECM) to facilitate capillary tube formation. Previously, we detected the presence of fibronectin fragments (Fn-f) associated with the proform of matrix metalloprotease-2 (MMP-2) in conditioned medium of human retinal endothelial cells (HRECs). Association of this fragment to latent MMP-2 prevented autocatalytic activation of MMP-2, suggesting a modulatory role of Fn-f in MMP-2 activation. In this report, we examined the potential role of Fn-f on two processes involved in angiogenesis, proliferation and migration of vascular cells. The effects of Fn-f on proliferation were determined by DNA synthesis and cell counts. Their effects on migration were assessed using modified Boyden chambers. Seven Fn-f were tested on vascular cell migration and/or proliferation. Three Fn-f induced migration. Fn-f of 30-kDa and 120-kDa size positively affected proliferation of microvascular cells but not macrovascular cells. A 45-kDa gelatin binding fragment of Fn inhibited HREC proliferation but stimulated pericyte and smooth muscle cell proliferation. The potency of these fragments exceeded that of the known angiogenic growth factor, basic fibroblast growth factor (bFGF), on HREC migration. ECM components such as fibronectin may influence capillary morphogenesis by the generation of fragments that can modulate proliferation, migration, and protease activation. In the setting of diabetes, excess Fn is generated and is available for degradation. Thus, the production of Fn-f may be specifically relevant to the angiogenesis observed in proliferative diabetic retinopathy. *Diabetes* **47:1335–1340, 1998**

Intrinsic to angiogenesis is the migration, proliferation, and formation of capillary tubes by endothelial cells. Quiescent endothelial cells become activated by soluble mitogens and insoluble extracellular matrix (ECM) molecules producing proteases for the degradation of matrix proteins to facilitate capillary tube formation (1). Fibronectin (Fn), a high-molecular-weight, adhesive, multifunctional glycoprotein and a key ECM component, has diverse biological activities (2). Fn exerts growth factor and differentiated activities in many types of cells and plays a vital role in cellular adhesion and migration, oncogenic transformation, wound healing, and hemostasis (3).

One of the most important functions of Fn is the maintenance of normal cell morphology via organization of cell attachment to the ECM. This is accomplished by a series of binding domains, including fibrin, factor XIIa, gelatin/collagen, DNA, heparin, and cell binding domains. The regions between these domains are highly susceptible to proteolysis, which gives rise to fibronectin fragments (Fn-f). Fn-f have been found to have activities not found in the intact molecule, and selected Fn-f have been shown to affect proliferation (4) and stimulate migration (5,6).

In a previous study, we found that human retinal endothelial cells (HRECs) of diabetic origin and nondiabetic HRECs after exposure to glucose expressed a novel proteolytic activity that migrated at 90 kDa. This 90-kDa activity represented the matrix metalloprotease-2 (MMP-2) tightly associated with Fn-f, and association of this fragment inhibited the autoactivation of MMP-2 (7). In the present study, the effect of selected Fn-f on vascular cell proliferation and migration was examined. This study supports that the generation of Fn-f may regulate microvascular cell behavior and that abnormal Fn-f formation in vivo could facilitate aberrant angiogenesis, as seen in proliferative diabetic retinopathy.

RESEARCH DESIGN AND METHODS

Cell cultures. Human eyes were obtained from the National Disease Resource Interchange within 36 h of death. The eyes were dissected, and the retinas were removed and digested. HREC cultures were prepared and the purity of the culture assessed as previously described (8). HRECs were routinely seeded at 6 × 10^3 cells/cm^2 in 75-cm^2 flasks. The cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2/95% air.

In certain studies, cells were treated with whole Fn (cellular Fn; Sigma, St. Louis, MO) from human foreskin fibroblasts or with the following Fn-f: 30-kDa tryptic (Sigma), which spans a small segment of the terminal end of the heparin II/fibrin I domain and continues into the collagen/gelatin binding domain; 40-kDa chymotryptic (Life Technologies, Gaithersburg, MD), comprising a small segment of the COOH-terminal end of the heparin I/cell binding domain and continues into the heparin III binding domain; 45-kDa tryptic (Sigma), which comprises most of the collagen/gelatin binding domain; 70-kDa cathepsin D (Sigma), which contains most of the heparin II/fibrin I as well as the collagen/gelatin binding domains; 110-kDa chymotryptic (Upstate Biotechnology, Lake Placid, NY), containing the cell binding (but not heparin I) domain; and 120-kDa chymotryptic (Life Technologies), which spans a small portion of the collagen/gelatin binding domain and nearly all of the heparin I/cell binding domain. All fragments were purified by high-performance liquid chromatography, reconstituted according to the manufacturer's instructions, and stored at −80°C in single-use aliquots. All fragments were analyzed by SDS-PAGE and confirmed as single bands by silver staining. To further confirm purity of Fn-f, NH_2-terminal sequencing was performed on the 120-kDa, 45-kDa, and 30-kDa proteins by the Protein Sequencing Core at the University of Florida.

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BFGF, basic fibroblast growth factor; BrdU, bromodeoxyuridine; DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; Fn, fibronectin; Fn-f, fibronectin fragment(s); HREC, human retinal endothelial cell; HUVEC, human umbilical vein endothelial cell; MMP-2, matrix metalloprotease-2; SMC, smooth muscle cell; tPA, tissue-type plasminogen activator.

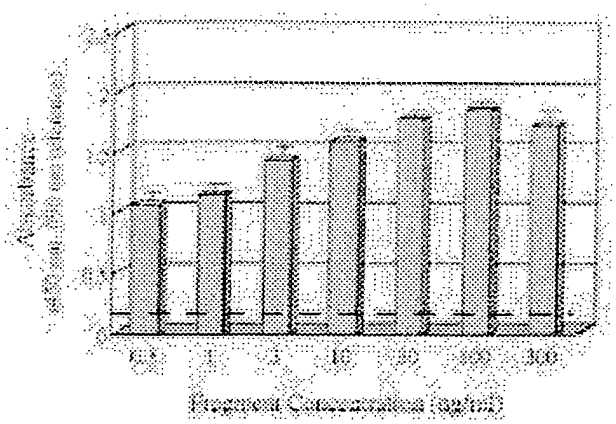

FIG. 1. HREC proliferation in response to increasing doses of the 120-kDa tryptic Fn-f. Cells were made quiescent by withdrawing serum for 18 h before the addition of 120-kDa Fn-f at the doses indicated. After an additional 24 h, the last 4 of which BrdU labeling reagent was added, the cells were processed according to manufacturer's directions to measure BrdU incorporation. Data are expressed as optical density at the wavelength indicated. The dashed line denotes the mean absorbance of untreated cells. Each bar is the mean of quadruplicate determinations, with standard error indicated by the error bar. BrdU incorporation reaches a plateau at 100 ng/ml ($P < 0.05$ for each dose up to 100 ng/ml vs. the previous dose; NS for 300 vs. 100 ng/ml).

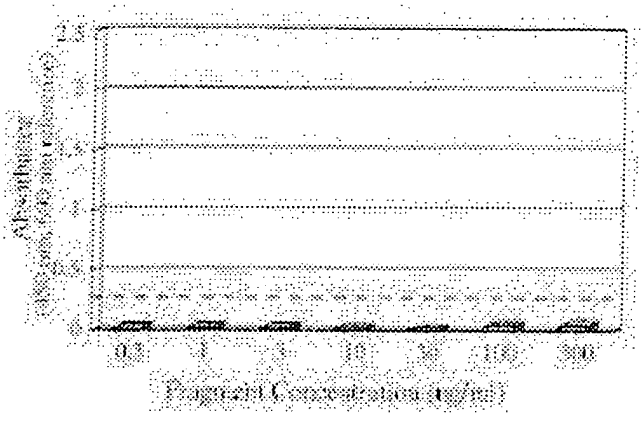

FIG. 2. Response of HUVECs to increasing doses of the 120-kDa chymotryptic Fn-f. These cells and the HRECs were treated identically. Data are expressed as optical density at the wavelength indicated. The dashed line denotes the mean absorbance of untreated cells. Each bar is the mean of quadruplicate determinations, with standard error indicated by the error bar. There is no dose-dependent trend in BrdU incorporation in these cells.

Human coronary artery smooth muscle cells (SMCs) were isolated and cultured as described (9). Human retinal pericytes were isolated at the time of HREC isolation and cultured separately as described (10). Human umbilical vein endothelial cells (HUVECs) were kindly provided by Dr. T. Maciag.

Proliferation. Bromodeoxyuridine (BrdU) incorporation (Boehringer Mannheim, Indianapolis, IN) was used to measure cell proliferation in response to various doses of Fn-f in HRECs, HUVECs, SMCs, and pericytes. Cells were grown in 96-well microtiter plates to ~50% confluence. They were then exposed to each Fn-f at the described doses for the indicated time of either 12 (HREC only), 24, 48, or 72 h. All treatments were performed in triplicate. Proliferation was indicated by a change in absorbance after reaction with anti-BrdU antibody and a colorimetric substrate reaction, as outlined by the manufacturer, and reported as fold change versus untreated cells. Based on the results of these studies, three Fn-f (30, 45, and 120 kDa) were selected for further examination as described below.

Migration studies. Chemotaxis studies were performed as previously described using modified Boyden chambers (11). For these studies, 25 µl of a suspension of HREC (1.6×10^6 cells/ml) was placed in each well of the inverted blindwell apparatus containing 48 wells. Wells were overlaid with a porous (5 µm diameter pores) polyvinyl-free and pyrrolidine-free polycarbonate membrane (Nuclepore, Pleasanton, CA), coated with 40 µg/ml bovine dermal collagen (Sigma). After allowing the cells to adhere to the membrane, chambers were then placed upright and test substances added (50 µl/well). Each Fn-f or whole Fn was tested at the indicated concentration. Dulbecco's modified Eagle's medium (DMEM) served as negative control, and DMEM containing 10% fetal calf serum served as positive control to assess chemotaxis. Chemokinesis, the nonoriented increase in cell locomotion in response to a stimulus, was measured for each Fn-f by adding equal concentrations of each fragment being tested to both upper and lower wells to abolish the concentration gradient. Chambers were disassembled after the specified time, cells on the attachment side were scraped off, and membranes were stained for analysis as previously described (11). Cells that migrated through the membrane were counted for each well and reported as cells per high power field. Additional chemotaxis experiments were performed comparing the effect of eqimolar concentrations of Fn-f and basic fibroblast growth factor (bFGF), since bFGF is a known stimulator of migration.

RESULTS

Proliferation. Of the five Fn-f examined, only three affected proliferation in HRECs. The 30-kDa and 120-kDa fragments induced proliferation as assessed by increased BrdU incorporation and cell number. Addition of the 120-kDa Fn-f resulted in a dose-dependent increase in BrdU incorporation in HRECs. Even at the lowest concentration examined, there was a 40-fold increase over basal, reaching maximal effect at 100 ng/ml (Fig. 1). When HUVECs were exposed to varying concentrations of the 120-kDa fragment, no change in BrdU incorporation was observed at any concentration tested (Fig. 2). The 30-kDa fragment resulted in a dose-dependent increase in BrdU incorporation. The maximal effect was achieved at 10 ng/ml. Higher concentrations resulted in stimulation, but not as great as at 10 ng/ml (Fig. 3). The 45-kDa fragment inhibited BrdU incorporation in HREC compared with untreated HREC (Fig. 3). However, the 45-kDa Fn-f increased BrdU incorporation in a dose-dependent manner in SMCs and pericytes (Fig. 4*A* and *B*). A maximal six- to sevenfold increase in BrdU incorporation was observed with both pericytes and SMCs, in contrast to the 20-fold decrease seen with this fragment in HRECs. The 30-kDa and the 120-kDa fragments increased BrdU incorporation

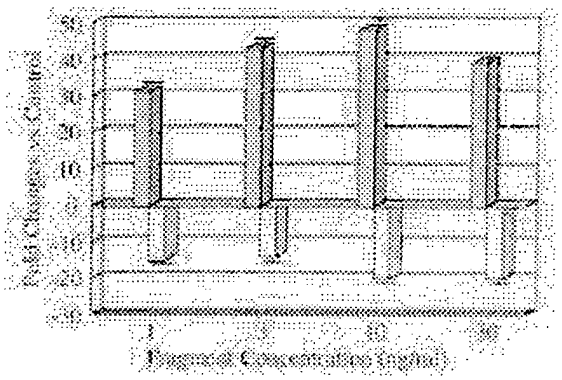

FIG. 3. HREC proliferation in response to increasing doses of the 30-kDa tryptic or 45-kDa tryptic Fn-f. Cells were treated identically to HRECs exposed to the 120-kDa Fn-f. Data were normalized to untreated cells and expressed as fold change. Each bar is the mean of quadruplicate determinations, with standard error indicated by the error bar. The 30-kDa fragment (▨) exhibits a dose-dependent increase in BrdU incorporation that reaches a plateau at 30 ng/ml ($P < 0.05$ for each dose up to 10 ng/ml vs. the previous dose; NS for 30 vs. 10 ng/ml). The 45-kDa fragment (□) exhibits a dose-dependent decrease in BrdU incorporation in these cells ($P < 0.05$ for each dose up to 30 ng/ml vs. the previous dose).

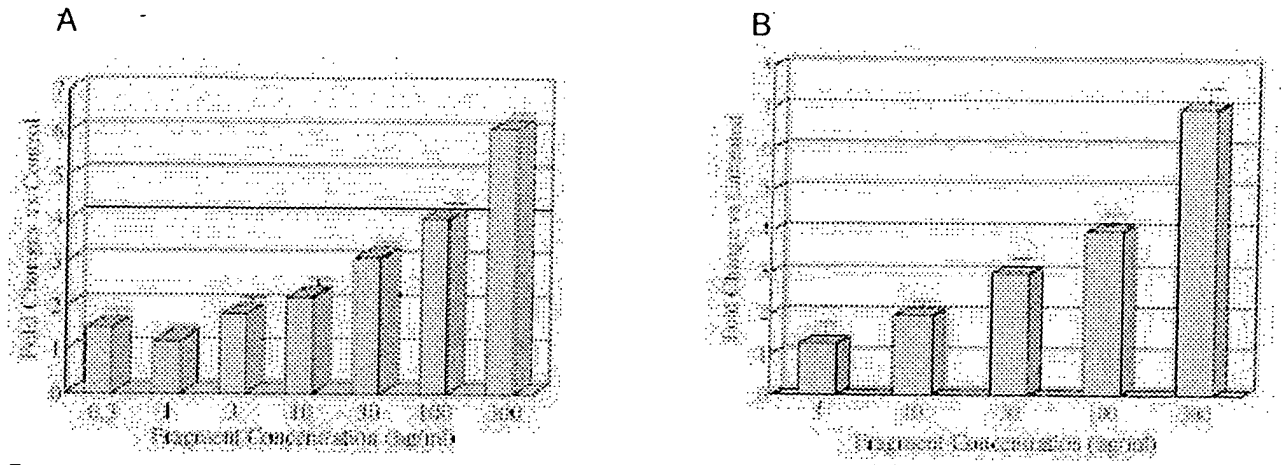

FIG. 4. Effect of increasing doses of the 45-kDa tryptic Fn-f on SMCs (*A*) or pericytes (*B*) in culture. Cells were treated identically to HRECs exposed to the 120-kDa Fn-f. Data were normalized to untreated cells and expressed as fold change. Each bar is the mean of quadruplicate determinations, with standard error indicated by the error bar. *A*: The 45-kDa Fn-f induces DNA synthesis in cultured SMCs in a dose-dependent fashion, showing a sixfold change versus untreated cells at the highest dose tested ($P < 0.05$ for each dose from 30 to 300 ng/ml vs. the previous dose; NS for 0.3 vs. 1 ng/ml). *B*: Cultured pericytes also show a dose-dependent increase in DNA synthesis in response to the 45-kDa Fn-f, with a sevenfold increase at the highest dose tested ($P < 0.05$ for each dose vs. the previous dose).

two- to threefold in SMC (data not shown) in contrast to the 35- to 70-fold increases observed in HREC.

The effect of exposure to Fn-f on cell number in HREC was examined. Cell number at 24 h rose in a dose-dependent manner by the addition of the 30-kDa and 120-kDa fragments. The addition of the 45-kDa fragment resulted in a dose-dependent decrease in cell number. At the highest concentration of 45-kDa Fn-f examined, cell number fell below that of wells containing medium alone (Fig. 5). The proliferative effect was evaluated only at 24 h exposure, because at 48–72 h the autocrine production of growth factors by these cells makes interpretation of the effect of the Fn-f difficult. The 120-kDa fragment and 30-kDa fragment did not stimulate an increase in cell number at 24 h in HUVEC (data not shown).

Migration studies. The three fragments examined above were tested in modified Boyden chambers. The 30-kDa fragment showed a dose-dependent increase in HREC migration (Fig. 6*A*). Similar results were observed with the 120-kDa fragment (Fig. 6*B*). The 45-kDa fragment also induced migration in a dose-dependent fashion (Fig. 6*C*). The chemotactic response observed with the 120-kDa fragment and the 45-kDa fragment exceeded the response observed with equal molar concentrations of intact Fn, whereas the effect of Fn on HREC migration was not significantly different from that observed with the 30-kDa Fn-f (data not shown). Interestingly, the response observed with the 120-kDa and 45-kDa Fn-f was greater than the response observed with equal molar concentrations of bFGF (Fig. 6*B* and *C*). Also, the fragments induced significant amounts of migration at 4 h of exposure, whereas the bFGF response required 8 h of exposure for optimal response in this system (data not shown). The chemokinetic effect of the 30-kDa, 45-kDa, and 120-kDa fragments was evaluated by checkerboard analysis and was not significant.

DISCUSSION

Capillary morphogenesis involves the orchestrated effects of growth factors that modulate endothelial cell proliferation, migration, and tube formation. Mesenchymal precursors are recruited to encase the endothelial tube. The precursors differentiate into pericytes, and there is inhibition of endothelial proliferation, resulting in a mature capillary. Growth factors and ECM proteins can modulate each of these steps in capillary formation.

The ECM protein Fn is present in the walls of vessels, is concentrated at pericyte endothelial contacts (12), and is also present in the internal limiting membrane of the adult retina. Although not all studies have been able to detect Fn in the basement membrane of capillaries, the inability to do so may have been due to the type of fixation used (12), to the fact that postembedding immunogold techniques were performed (13), or to the fact that antigen retrieval techniques needed for many basement membrane proteins were not used. Others who did find Fn used a variety of more sensitive

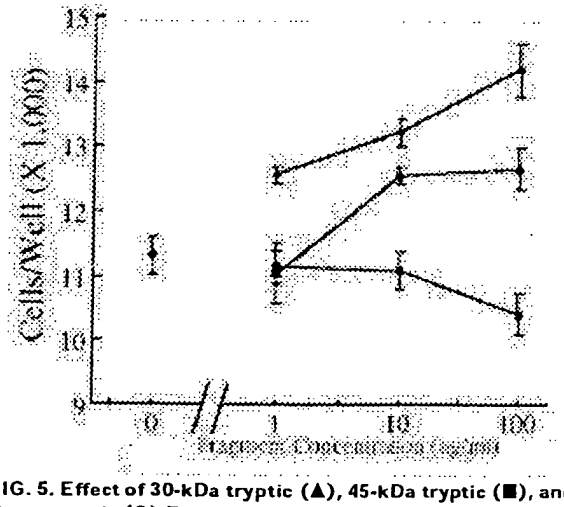

FIG. 5. Effect of 30-kDa tryptic (▲), 45-kDa tryptic (■), and 120-kDa chymotryptic (●) Fn-f on HREC proliferation as measured by change in cell number. Cells were plated in 24-well multiwell plates, then made quiescent by withdrawing serum for 18 h before adding Fn-f at the doses indicated. Serum-free medium was used as negative control (◆). After an additional 24 h of incubation, the cells were enzymatically dissociated and each well counted separately using a Coulter counter. Each point is the mean of four determinations. Error bars show standard error. Both the 30-kDa and 120-kDa Fn-f induce cell proliferation, whereas the 45-kDa fragment inhibits proliferation.

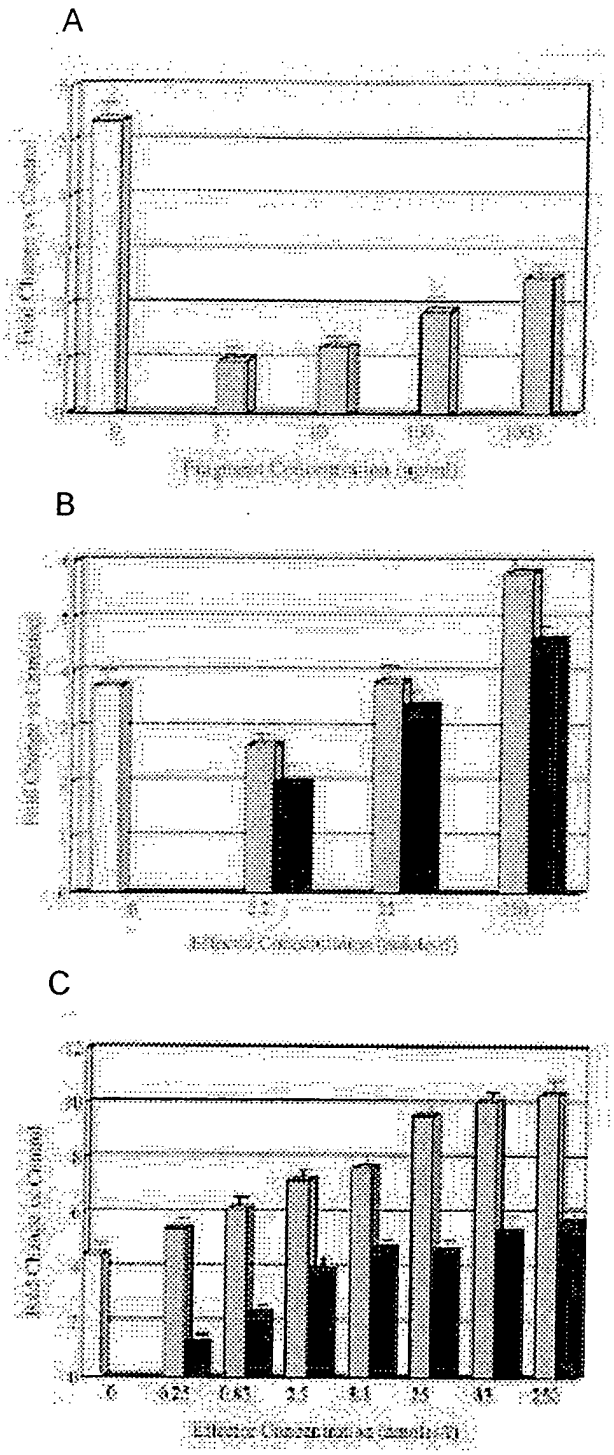

FIG. 6. Effect of Fn-f on migration of HREC, as measured using modified Boyden chambers. All three fragments induced dose-dependent migration of cultured microvascular endothelial cells. In all cases, medium containing 10% (wt/vol) fetal bovine serum was used as positive control. Each bar represents the mean of eight determinations, with error bars denoting standard error. All data were normalized to unstimulated cells (media alone) and expressed as fold change in the number of cells migrating. *A*: The 30-kDa fragment induces up to a 2.5-fold increase in migration at the highest dose tested, about half that induced by media + fetal bovine serum (□ on all three parts). The dose-dependent migration induced by either the 45-kDa (*B*, ▨) or the 120-kDa (*C*, ▨) fragments is actually greater (at maximally tested doses) than that induced by either media + fetal bovine serum or equal molar concentrations of bFGF (*B* and *C*, ■). In all cases, the data represented by each bar are significantly different ($P < 0.05$) from the previous dose.

techniques, including immunofluorescence and immunoperoxidase (14,15).

Immunofluorescence studies after trypsin digestion of the retina showed increased Fn immunoreactivity in large vessels and microvessels of patients with diabetes compared with control subjects. In situ hybridization studies of the trypsin-digested retinas from these diabetic patients showed Fn mRNA in the retina and retinal microvessels, a direct argument in support of local synthesis of Fn in the vessels of the human retina (16).

Using electron microscopy immunogold studies, we observed increases in Fn immunoreactivity in capillary basement membranes of rats with spontaneous diabetes compared with age-matched nondiabetic controls (17). In addition, the basement membrane zone of many new blood vessels is positive for an oncofetal Fn isoform containing the ED-B domain, a marker of angiogenesis (18,19).

Previous studies from our laboratory have shown that HRECs of diabetic origin expressed increased amounts of Fn compared with HRECs of nondiabetic origin. Exposure of HRECs of nondiabetic origin to high glucose increased the amount of Fn protein expressed (20). The diabetes-induced overexpression of Fn by endothelial cells is not readily reversible, and high glucose can mimic this effect in endothelial cells of nondiabetic origin. After six or seven cell replications, the glucose-induced elevation in Fn and type IV collagen is greater than in control cells (21). The differences we observed in HRECs of diabetic and nondiabetic origin give support to the finding that the events occurring during a finite period of metabolic derangement can leave long-lasting sequelae in the system (8). Processes that could propagate a "memory" of the diabetic state include hyperglycemia-induced irreversible modifications of long-lived ECM proteins. Although some effects of diabetes can be reversed by adequate insulinization, Fn overexpression and increased synthesis of glomerular basement membrane collagen do not decrease with treatment of diabetes. Increased Fn synthesis is also observed in fibroblasts explanted from diabetic mice and passaged in culture (22).

Proteolysis of Fn occurs near cells undergoing neoplastic transformation (23). Tumor cells elaborate proteases that can cleave Fn, including plasmin (24–26). Fn-f have been identified at sites of inflammation, injury, and destruction by metastatic tumor cells (27–31). Fn-f have been found to have activities not found in the intact molecule. Selected Fn-f have been shown to affect proliferation (27,28) and promote the adhesion (30,31), spreading, and migration of vascular endothelial cells (4–6,31,32).

Fn-f also induce expression of various proteases, including elastase (33), stromelysin (34), and metalloproteases (35,36). Studies by Imhoff et al. (37) demonstrated that a 190-kDa fragment of Fn produced by cathepsin D proteolysis in the presence of Ca^{2+} undergoes spontaneous autolysis, generating two enzymes, Fn-gelatinase and Fn-laminase, specific for the degradation of the ECM proteins, laminin, and Fn.

Recent studies have shown that Fn-f derived from residues 196–203 are potent stimulators of plasminogen activation catalyzed by tissue-type plasminogen activator (tPA) (38). Fn-f increased the efficacy of the plasminogen substrate. This region of Fn was within the fifth type-1 repeat in the NH_2-terminal domain of Fn. The primary physiological role of type-1 repeats is to bind fibrin, which

enhances the catalytic activity of tPA. Thus, interaction of plasminogen and tPA with ECM components may provide a fine regulatory mechanism for localized generation of plasmin proteolytic activity within the ECM.

Previous studies in our laboratory showed that HRECs express tPA in the quiescent state and urokinase when wounded in culture, and that plasmin activity was easily detectable in the conditioned medium of HRECs under basal conditions (8). The generation of plasmin can lead to activation of proforms of MMPs, and plasmin can degrade Fn, as can MMP-2. We have recently shown that HRECs produce MMP-2 (7). Fn-f could be generated in vivo at sites of angiogenesis by proteases secreted by endothelial cells (e.g., plasmin and MMPs) and/or mast cells (e.g., tryptase) (39).

The present study shows that the 120-kDa fragment increases DNA synthesis and cell number, but only in capillary endothelial cells, as this effect was not observed in HUVEC. The effect of the 30-kDa Fn-f is also specific to endothelial cells of the microvasculature, as the fragment did not affect HUVEC proliferation. We observed that selected fragments (45-kDa Fn-f) had opposite effects on HRECs and pericytes, a response that would be particularly beneficial if inhibition of endothelial cell proliferation was desired at the same time pericyte proliferation was required. For example, during the final stages of angiogenesis, the endothelial tube is already formed, but pericytes must still migrate and proliferate to encase this endothelial tube to complete the formation of the capillary. We have also observed that the Fn-f are more potent and act more quickly than bFGF on endothelial cell migration.

In summary, this study and previous work from our laboratory provide unique evidence for specific Fn-f regulating both cell movement and cell proliferation and activation of latent MMP-2 (7). Fn and Fn-f have potentially competing actions on steps relevant to angiogenesis. These studies support a complex regulatory role of Fn and its various domains, obtained by proteolytic degradation of Fn, in angiogenesis. The modulation by glucose of ECM components, such as Fn, may modify cellular behavior that may be specifically relevant to angiogenesis in diabetic retinopathy.

ACKNOWLEDGMENTS

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REFERENCES

1. Ingber D: Extracellular matrix and cell shape: potential control points for inhibition of angiogenesis. *J Cell Biochem* 47:236–241, 1991
2. Liotta LA, Tryggyason K, Garbisa S, Hart I, Foltz CM, Shafie S: Metastatic potential correlates with enzymic degradation of basement membrane collagen. *Nature* 284:67–68, 1980
3. Ruoslahti E: Fibronectin and its receptors. *Annu Rev Biochem* 57:375–413, 1988
4. Humphries MJ, Ayad SR: Stimulation of DNA synthesis by cathepsin D digests of fibronectin. *Nature* 305:811–813, 1983
5. Postlethwaite AE, Keski-Oja J, Balian G, Kang AH: Induction of fibroblast chemotaxis by fibronectin: localization of the chemotactic region to a 140,000-molecular weight non-gelatin-binding fragment. *J Exp Med* 153:494–499, 1981
6. Tsukamoto Y, Helsel WE, Wahl SM: Macrophage production of fibronectin, a chemoattractant for fibroblasts. *J Immunol* 127:673–678, 1981
7. Grant MB, Caballero S, Tarnuzzer RW, Bass KE, Ljubimov A, Spoerri PE, Galardy RE: Matrix metalloproteinase expression in cultured human retinal microvascular cells. *Diabetes* 47:1317–1323, 1998
8. Grant MG, Guay C: Plasminogen activator production by human retinal endothelial cells of nondiabetic and diabetic origin. *Invest Ophthalmol Vis Sci* 32:53–64, 1991
9. Grant MB, Wargovich TJ, Ellis EA, Caballero S, Mansour M, Pepine CJ: Localization of insulin-like growth factor I and inhibition of coronary smooth muscle cell growth by somatostatin analogues in human coronary smooth muscle cells: a potential treatment for restenosis? *Circulation* 89:1511–1517, 1994
10. Antonelli-Orlidge A, Saunders KB, Smith SR, D'Amore PA: An activated form of transforming growth factor beta is produced by cocultures of endothelial cells and pericytes. *Proc Natl Acad Sci U S A* 86:4544–4548, 1989
11. Grant M, Jerdan J, Merimee TJ: Insulin-like growth factor-I modulates endothelial cell chemotaxis. *J Clin Endocrinol Metab* 65:370–371, 1987
12. Courtoy PJ, Boyles J: Fibronectin in the microvasculature: localization in the pericyte-endothelial interstitium. *J Ultrastruct Res.* 83:258–273, 1983
13. Das A, Frank RN, Zhang NL, Turczyn TJ: Ultrastructural localization of extracellular matrix components in human retinal vessels and Bruch's membrane. *Arch Ophthalmol* 108:421–429, 1990
14. Essner E, Lin WL: Immunocytochemical localization of laminin, type IV collagen and fibronectin in rat retinal vessels. *Exp Eye Res* 47:317–327, 1988
15. Jerdan JA, Glaser BM: Retinal microvessel extracellular matrix: an immunofluorescent study. *Invest Ophthalmol Vis Sci* 27:194–203, 1986
16. Roy S, Cagliero E, Lorenzi M: Fibronectin overexpression in retinal microvessels of patients with diabetes. *Invest Ophthalmol Vis Sci* 37:258–266, 1996
17. Ellis E, Grant M, Murray F, Wachowski M, Gubersky DL, Kubilis PS, Lutty GA: Increased NADH oxidase activity in the retina of the BBZ/Wor diabetic rat. *Free Rad Biol Med* 24:111–120, 1998
18. Ljubimov AV, Burgeson RE, Butkowski RJ, Couchman JR, Zardi L, Ninomiya Y, Sado Y, Huang ZS, Nesburn AB, Kenney MC: Basement membrane abnormalities in human eyes with diabetic retinopathy. *J Histochem Cytochem* 44:1469–1479, 1996
19. Castellani P, Viale G, Dorcaratto A, Nicolo G, Kaczmarek J, Querze G, Zardi L: The fibronectin isoform containing the ED-B oncofetal domain: a marker of angiogenesis. *Int J Cancer* 59:612–618, 1994
20. Munjal ID, McLean NV, Blake DA, Grant MB: Differences in the synthesis of secreted proteins in human retinal endothelial cells of diabetic and nondiabetic origin. *Curr Eye Res* 13:303–310, 1994
21. Roy S, Sala R, Cagliero E, Lorenzi M: Overexpression of fibronectin induced by diabetes or high glucose: phenomenon with a memory. *Proc Natl Acad Sci U S A* 87:404–408, 1990
22. Phan-Thanh L, Robert L, Derouette JC, Labat-Robert J: Increased biosynthesis and processing of fibronectin in fibroblasts from diabetic mice. *Proc Natl Acad Sci U S A* 84:1911–1915, 1987
23. Vaheri A, Mosher DF: High molecular weight, cell surface-associated glycoprotein (fibronectin) lost in malignant transformation. *Biochim Biophys Acta* 516:1–25, 1978
24. van-Muijen GN, Danen EH, de-Vries TJ, Quax PH, Verheijen JH, Ruiter DJ: Properties of metastasizing and nonmetastasizing human melanoma cells. *Recent Results Cancer Res* 139:105–122, 1995
25. Chen WT, Lee CC, Goldstein L, Bernier S, Liu CH, Lin CY, Yeh Y, Monsky WL: Membrane proteases as potential diagnostic and therapeutic targets for breast malignancy. *Breast Cancer Res Treat* 31:217–226, 1994
26. Gingras MC, Jarolim L, Finch J, Bowden GT, Wright JA, Greenberg AH: Transient alterations in the expression of protease and extracellular matrix genes during metastatic lung colonization by H-ras-transformed 10T1/2 fibroblasts. *Cancer Res* 50:4061–4066, 1990
27. Preciado-Patt L, Levartowsky D, Prass M, Hershkoviz R, Lider O, Fridkin M: Inhibition of cell adhesion to glycoproteins of the extracellular matrix by peptides. *Eur J Biochem* 223:35–42, 1994
28. Mortarini R, Gismondi A, Santoni A, Parmiani G, Anichini A: Role of the alpha 5 beta 1 integrin receptor in the proliferative response of quiescent human melanoma cells to fibronectin. *Cancer Res* 52:4499–4506, 1992
29. Yoshihara T, Ikushima S, Shimizu Y, Esumi N, Todo S, Humphries MJ, Imashuku S: Distinct mechanism of human neuroblastoma cell adhesion to fibronectin. *Clin Exp Metastasis* 9:363–375, 1991
30. Saiki I, Makabe T, Yoneda J, Murata J, Ishizaki Y, Kimizuka F, Kato I, Azuma I: Inhibitory effect of fibronectin and its recombinant polypeptides on the adhesion of metastatic melanoma cells to laminin. *Jpn J Cancer Res* 82:1112–1119, 1991
31. Makabe T, Saiki I, Murata J, Ohdate Y, Kawase Y, Taguchi Y, Shimojo T, Kimizuka F: Modulation of haptotactic migration of metastatic melanoma cells by the interaction. *J Biol Chem* 265:14270–14276, 1990
32. Huebsch JC, McCarthy JB, Diglio CA, Mooradian DL: Endothelial cell interactions with synthetic peptides from the carboxyl-terminal heparin-binding

domains of fibronectin. *Circ Res* 77:43–53, 1995
33. Xie D, Hui F, Homandberg GA: Fibronectin fragments alter matrix protein synthesis in cartilage tissue cultured in vitro. *Arch Biochem Biophys* 307:110–118, 1993
34. Muir D, Manthorpe M: Stromelysin generates a fibronectin fragment that inhibits Schwann cell proliferation. *J Cell Biol* 116:177–185, 1992
35. Werb Z, Hembry R, Murphy G, Aggeler J: Commitment to expression of the metalloendopeptidases, collagenase and stromelysin: relationship of inducing events to changes in cytoskeletal architecture. *J Cell Biol* 102:697–702, 1986
36. Aggeler J, Frisch SM, Werb Z: Changes in cell shape correlate with collagenase gene expression in rabbit synovial. *J Cell Biol* 98:1662–1671, 1984
37. Imhoff JM, Blondeau X, Planchenault T, Emod I, Keil-Dlouha V: Collagenase activation of latent matrix-degrading proteinases from human plasma. *Biol Chem Hoppe Seyler* 371:137–144, 1990
38. Stack S, Gray RD, Pizzo SV: Modulation of plasminogen activation and type IV collagenase activity by a synthetic. *Biochemistry* 30:2073–2077, 1991

Gluconeogenesis, glucose ...

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Gluconeogenesis, glucose production and fasting glycaemia

Original article:
Influence of obesity and type 2 diabetes on gluconeogenesis and glucose output in humans. A quantitative study. Gastaldelli A, Baldi S, Pettiti M et al. Diabetes 2000; 49: 1367-73.

Summary

Increased endogenous (hepatic and possibly renal) glucose production is thought to be a major factor regulating fasting glycaemia in Type 2 diabetes. Whether it relates to enhanced gluconeogenesis, elevated glycogenolysis, or both, is debatable. Little information is available on the effects of obesity per se on endogenous glucose production.

In the present study, fasting endogenous glucose fluxes and glycaemia were measured in 40 obese subjects (28 with Type 2 diabetes and 12 non-diabetic controls) and 15 non-obese subjects (nine with Type 2 diabetes and six non-diabetic controls). Endogenous glucose production was measured using 6,6-di-deuterated glucose, and gluconeogenesis by the 2H2O technique [1].

Several interesting observations were made. First, gluconeogenesis was increased in obese non-diabetic subjects. Second, gluconeogenesis was markedly increased in both obese and non-obese diabetic subjects. Third, gluconeogenesis and endogenous glucose production were both positively correlated with fasting glycaemia (Fig. 1). And, finally, hyperglucagonaemia was present in diabetic patients and was positively correlated with glucose fluxes.

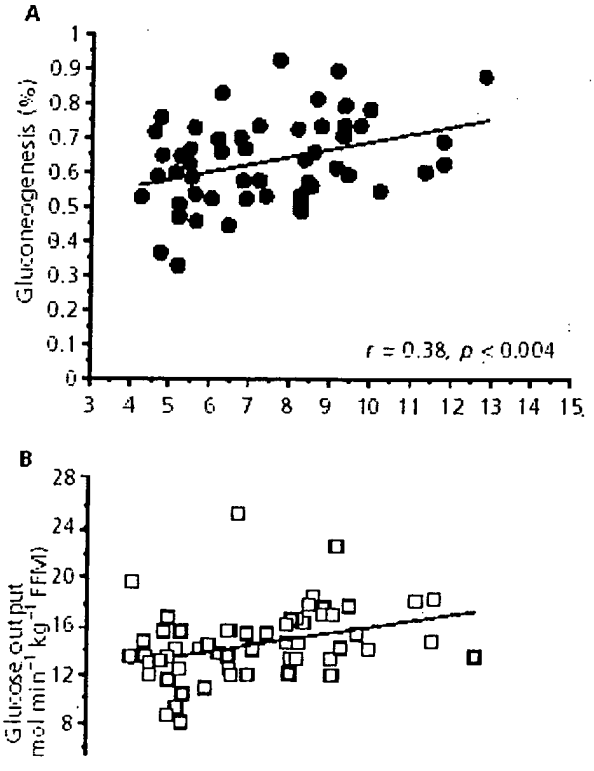

Fig. 1: Linear relationship between fasting plasma glucose and percent gluconeogenesis (A) and endogenous glucose output (B).

Comment

The fact that increased glucose production plays an important role in the pathogenesis of fasting hyperglycaemia in Type 2 diabetes is well documented. However, the mechanisms responsible for this increased glucose production are unknown. Several factors may be involved, including hepatic or renal resistance to the suppressive actions of both glucose and insulin on glucose production, increased secretion of

counterregulatory hormones, intrinsic dysregulation of hepatic/renal pathways of glucose production, or extrahepatic consequences of insulin resistance. Among the latter, increased lipolysis and proteolysis may stimulate gluconeogenesis by increasing gluconeogenic substrate availability and by enhancing plasma free fatty acid concentrations. High plasma free fatty acid concentration may in turn enhance hepatic gluconeogenesis and glucose output [2].

Obesity per se is also characterized by elevated rates of lipolysis and hence of glycerol and fatty acid release by adipose tissue. It may therefore be a major factor in enhanced glucose production under special circumstances. The present study carefully measured glucose production and gluconeogenesis in a large group of subjects including obese and non-obese individuals. The several pitfalls associated with measurements of glucose fluxes were skilfully avoided. The results obtained in obese subjects corroborate our earlier observations, i.e. that obesity per se stimulates gluconeogenesis without altering total glucose output [3, 4]. It has been recognized for several years that stimulation of gluconeogenesis can be attained by infusion of gluconeogenic precursors such as glycerol [5] or lactate [6]. Under such circumstances, an autoregulatory mechanism within the liver appears to prevent an increase in glucose production by simultaneously reducing net glycogenolysis [7]. This study clearly indicates that this autoregulatory mechanism is intact in obese non-diabetic patients.

In obese Type 2 diabetics, the picture, however, was quite different. Gluconeogenesis was further enhanced, possibly related to more severe extrahepatic and hepatic insulin resistance, and resulted in an increased glucose-6-phosphate flux in glucose-producing cells. Furthermore, hyperglucagonaemia was present, and is likely to play a major role in increasing glucose production, possibly by stimulating glucose-6-phosphatase. Last, but not least, both increased glucose production and whole body insulin resistance (documented by lower glucose clearance in diabetic patients) contributed to the development of hyperglycaemia.

What can we learn from these data? First, increased gluconeogenesis is not a sufficient factor per se to produce fasting hyperglycaemia. This suggests that strategies aimed at reducing gluconeogenesis may be ineffective in reducing glycaemia in Type 2 diabetes. This conclusion appears to be supported by the observation that acute ethanol administration did not lower glycaemia, although it suppressed gluconeogenesis in Type 2 patients [8]. Second, additional factors are present in Type 2 diabetes to stimulate glucose production. Hyperglucagonaemia may be one such factor, but other hormones or cytokines as well as neural factors may also be involved. Identification of such factors and of their mode of action may point to novel potential therapeutic strategies to reduce glucose production and fasting hyperglycaemia in Type 2 diabetes.

References

1. Landau BR, Wahren J, Chandramouli V et al. Use of 2H2O for estimating rates of gluconeogenesis. J Clin Invest 1995; 95: 172-8.
2. Rebrin K, Steil GM, Getty L, Bergman RN. Free fatty acid as a link in the regulation of hepatic glucose output by peripheral insulin. Diabetes 1995; 44: 1038-45.
3. Müller C, Mosimann F, Assimacopoulos-Jeannet F et al. Endogenous glucose production, gluconeogenesis and liver glycogen concentration in obese non-diabetic patients.

Diabetologia 1997; 40: 463-8.
4. Giusti V, Schneiter P, Thiébaud D et al. Influences of body weight, body composition, and substrate oxidation rate on resting postabsorptive glucose production and gluconeogenesis. Int J Obesity 1996; 20: 842-7.
5. Winkler B, Rathgeb I, Steele R, Altszuler N. Conversion of glycerol to glucose in the normal dog. Am J Physiol 1970; 219: 497-502.
6. Jenssen T, Nurjhan N, Consoli A, Gerich JE. Failure of substrate-induced gluconeogenesis to increase overall glucose appearance in normal humans. J Clin Invest 1990; 86: 489-97.
7. Tappy L, Jéquier E, Schneiter P. Autoregulation of glucose production. News Physiol Sci 2000; 15: 198-202.
8. Puhakainen I, Koivisto VA, Yki-Järvinen H. No reduction in total hepatic glucose output by inhibition of gluconeogenesis with ethanol in NIDDM patients. Diabetes 1991; 40: 1319-27.

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Luc Tappy, Lausanne, Switzerland

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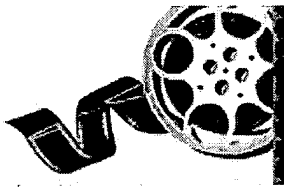

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mark·er **Pronunciation Key** (mär'kər)
n.

1. One that marks or serves as a mark, as:
   a. A bookmark.
   b. A tombstone.
   c. A milestone.
2. An implement, especially a felt-tipped pen, used for marking or writing.
3. One who marks objects, especially for industrial purposes.
4. One who grades student papers.
5. *Sports.*
   a. A device, such as a line, stake, or flag, set on a playing field and showing the playing or scoring position.
   b. A player who guards an opponent, as in soccer.
   c. An official in certain court games, such as squash, who mainly judges whether the ball is out of play and whether a fault or let has occurred.
6. *Games.*
   a. One that keeps score in various games.
   b. A score in a game.
7. *Slang.* A written, signed promissory note.

8. A genetic marker.
9. *Medicine.* A physiological substance, such as human chorionic gonadotropin or alpha-fetoprotein, that when present in abnormal amounts in the serum may indicate the presence of disease, as that caused by a malignancy. Also called **biomarker**.
10. *Linguistics.* An element that indicates grammatical class or function; a derivational or inflectional morpheme.

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mark·er (mär'kər)
n.

1. One that marks or serves as a mark.
2. A physiological substance, such as human chorionic gonadotropin or alpha-fetoprotein, that may indicate disease when present in abnormal amounts in the serum, as that caused by a malignancy. Also called *biomarker.*
3. A genetic marker.

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Main Entry: **mark·er**
Pronunciation: 'mär-k&r
Function: *noun*
1 : something that serves to identify, predict, or characterize <a surface *marker* on a cell that acts as an antigen> <erythema migrans ... is a distinctive *marker* for Lyme disease —Cecelia E. Holmes & Mary C. Massa>
2 : GENETIC MARKERcalled also *marker gene*

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marker

n 1: some conspicuous object used to distinguish or mark something; "the buoys were markers for the channel" 2: a distinguishing symbol; "the owner's mark was on all the sheep" [syn: marking, mark] 3: a writing implement for making a mark

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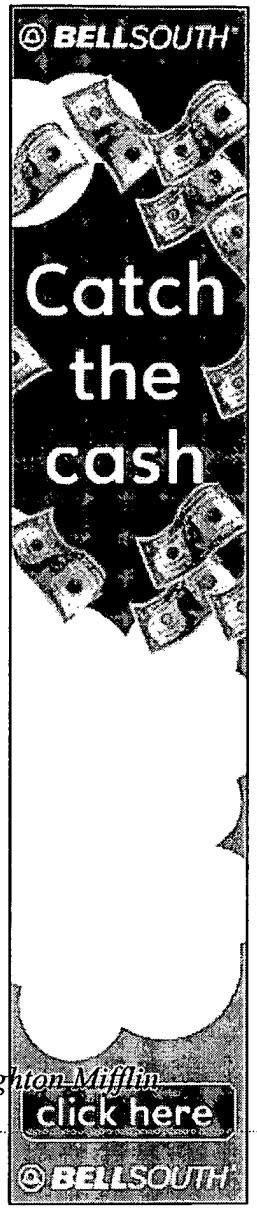

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Elevated 5-S-cysteinyldopamine/homovanillic acid ratio and reduced homovanillic acid in cerebrospinal fluid: possible markers for and potential insights into the pathoetiology of Parkinson's disease.

Cheng FC, Kuo JS, Chia LG, Dryhurst G.

Department of Medical Research and Geriatrics Medical Center, Taichung, Taiwan, Republic of China.

High-performance liquid chromatography with electrochemical detection has been employed to analyze ultrafiltrates of cerebrospinal fluid of Parkinson's Disease (PD) patients and age-matched controls for the dopamine (DA) metabolites homovanillic acid (HVA) and 5-S-cysteinyldopamine (5-S-CyS-DA). The mean level of HVA in the CSF of PD patients, measured 5 days after withdrawal from L-DOPA therapy, was significantly lower than that measured in controls. By contrast, mean levels of 5-S-CyS-DA were not significantly different in the CSF of PD patients taking L-DOPA (PD-LT patients) the same patients 5 days after discontinuing this drug (PD-LW patients) or controls. However, the mean 5-S-CyS-DA/HVA concentration ratio was significantly ($p < 0.05$) higher in the CSF of PD-LW patients compared to controls. Although the PD patient population employed in this study had been diagnosed with the disease several years previously and had been treated with L-DOPA for prolonged periods of time the results of this study suggest that low CSF levels of HVA and a high 5-S-CyS-DA/HVA ratio together might represent useful markers for early diagnosis of PD. The high 5-S-CyS-DA/HVA ratio observed in the CSF of PD-LW patients also provides support for the hypothesis that the translocation of glutathione or L-cysteine into neuromelanin-pigmented dopaminergic cell bodies in the substantia nigra might represent an early event in the pathogenesis of PD.

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Chromogranin A in cerebrospinal fluid: a biochemical marker for synaptic degeneration in Alzheimer's disease?

Blennow K, **Davidsson P**, **Wallin A**, **Ekman R**.

Department of Psychiatry and Neurochemistry, University of Goteborg, Molndal Hospital, Sweden.

Biochemical markers for AD would be of great value both to improve the clinical diagnostic accuracy in scientific studies and to increase the knowledge of the pathogenesis of the disorder. One of the main features of AD is a degeneration of synapses. Therefore, we examined if chromogranin A (CrA), the major protein of large dense-core synaptic vesicles, in cerebrospinal fluid (CSF) may be of value as a biochemical marker for the synaptic function in AD. The mean concentration of CrA in CSF was about 7.5 times higher than its concentration in serum, and there was no significant correlation between CSF-CrA and the blood-brain barrier function (measured as the CSF/serum albumin ratio), nor between CSF-CrA and serum-CrA. These findings suggest that the major portion of CSF-CrA is locally produced within the CNS. There were no significant differences in CSF-CrA between the AD (n = 29), vascular dementia (n = 13), and age-matched control (n = 9) groups (99.9 +/- 58.9 ng/ml, 108.0 +/- 69.4 ng/ml, and 115.1 +/- 44.4 ng/ml, respectively). However, when the AD group was subdivided into AD type I (n = 12) and AD type II (n = 17), a lower concentration of CSF-CrA was found in AD type I (72.8 +/- 28.9 ng/ml) compared with controls (115.1 +/- 44.4 ng/ml), $p < 0.02$, and compared with AD type II (119.1 +/- 67.5 ng/ml), $p < 0.05$, while CSF-CrA did not significantly differ between AD type II and controls. These findings suggest that CSF-CrA has a potential as a biochemical marker for the synaptic degeneration in AD type I, and gives further support for the relevance of identifying the AD type I (pure AD) subgroup in scientific studies.

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n.

One of the rings or loops forming a chain.

A unit in a connected series of units: *links of sausage; one link in a molecular chain.*

A unit in a transportation or communications system.

A connecting element; a tie or bond: *grandparents, our link with the past.*

An association; a relationship: *The Alumnae Association is my link to the school's present administration.*

A causal, parallel, or reciprocal relationship; a correlation: *Researchers have detected a link between smoking and heart disease.*

A cuff link.

Abbr. **li** A unit of length used in surveying, equal to 0.01 chain, 7.92 inches, or about 20.12 centimeters.

A rod or lever transmitting motion in a machine.

Computer Science. A segment of text or a graphical item that serves as a cross-reference between parts of a hypertext document or between files or hypertext documents. Also called **hotlink, hyperlink**

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Mass spectrometry and proteomics

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SCOTT D. PATTERSON
Amgen Inc., Thousand Oaks, California 91320-1789

THE 15TH ASILOMAR CONFERENCE on Mass Spectrometry this October was devoted to the role of mass spectrometry (MS) in proteomics. The Asilomar Conference site is in a picturesque national park in Pacific Grove, CA, overlooking the Pacific Ocean. The conference aims to bring together scientists from a cross section of disciplines that are applying MS to an emerging field. This year, that emerging field is proteomics. The term "proteome" was coined by Wilkins et al. (17) in the mid-1990s to describe the protein complement of the genome. The term was first used to describe the 20-yr-old field of two-dimensional gel electrophoresis (2-DE) and quantitative image analysis. 2-DE remains the highest resolution protein separation method available, but the ability to identify the observed proteins was always an extremely difficult problem. MS has been integral to solving that problem. Although improvements in 2-D gel technology had been realized since its introduction, three enabling technological advances have provided the basis for the foundation of the field of proteomics. The first advance was the introduction of large-scale nucleotide sequencing of both expressed sequence tags (ESTs) and, more recently, genomic DNA. The second was the development of mass spectrometers able to ionize and mass-analyze biological molecules and, more recently, the wide-spread introduction of mass spectrometers capable of data-dependent ion selection for fragmentation (MS/MS) (i.e., without the need for user intervention). The third was the development of computer algorithms able to match uninterpreted (or partially interpreted) MS/MS spectra with translations of the nucleotide sequence databases, thereby tying the first two technological advances together. Thus MS played a key role in the passage of 2-DE/image analysis to proteomics.

As a note to readers unfamiliar with MS, the instruments are named for their type of ionization source and mass analyzer (see also Refs. 1, 11, 12). To measure the mass of molecules, the test material must be charged (hence ionized) and desolvated (dry). The two most successful mechanisms for ionization of peptides and proteins are matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI). In MALDI the analyte of interest is embedded in a matrix that is dried and then volatilized in a vacuum under ultraviolet laser irradiation. This is a relatively efficient process that ablates only a small portion of the analyte with each laser shot. Typically, the mass analyzer coupled with MALDI is a time-of-flight (TOF) mass analyzer that simply measures the elapsed time from acceleration of the charged (ionized) molecules through a field-free drift region. The other common ionization source is ESI, in which the analyte is sprayed from a fine needle at high voltage toward the inlet of the mass spectrometer (which is under vacuum) at a lower voltage. The spray is typically either from a reversed-phase HPLC (RP-HPLC) column or a nanospray device (19) that is similar to a microinjection needle. During this process, the droplets containing analyte are dried and gain charge (ionize). The ions formed during this process are directed into the mass analyzer, which could be either a triple-quadrupole, an ion trap, a Fourier-transform ion cyclotron resonance (FT-ICR), or a hybrid quadrupole TOF (Qq-TOF) type.

This Asilomar meeting provided one of the largest academic forums in the United States for the presentation and discussion of MS as it is applied to proteomics. As is obvious from the introduction, the initial role MS played was as a protein identification and characterization methodology. However, the role of MS is expanding in this field. Although a series of talks focused on the use of different kinds of MS to identify gel-separated proteins and the various automation technologies applied to perform this in high throughput, several talks also presented alternate approaches. These approaches utilized direct analysis of digested protein mixtures for either identification of the components or quantitative analysis of two different samples mixed together. Specific biological applications were also presented. As described above, a critical component of any MS approach as applied to proteomics is the computational analysis. This report will be divided to focus on these six aspects of MS in proteomics. Where references are known for some of the material presented, they are cited. The program was, however, not entirely limited to MS in proteomics. Prior to the six sections covering the conference core, the first section of this report covers those presentations that were aimed at providing an insight into broader biological and drug discovery processes.

Proteomics in biology and drug discovery. The opening lecture, given by Lee Hood (Univ. of Washington), provided an excellent overview of Genomics, Proteomics, and Systems Biology. Hood described the genome project efforts that provide four types of maps: genetic, physical, gene, and sequence. For the human genome,

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it is anticipated that 90–95% of all genes will be sequenced sometime next year. This is the first step toward what Hood described as the "Periodic Table of Life." The different approaches to genomic sequencing and microarray technologies that are able to interrogate the mRNA levels of thousands of genes at a time were described. Hood described proteomics in broad terms as the study of multiplicity of proteins. The information obtained from the various hierarchical levels of biological information (gene, protein, pathways, interconnecting pathways) must be integrated for us to be able to provide a more complete biological picture. For both microarray and proteomics, samples representing the disease process must be obtained. This means that pure cell populations must be microscopically captured from tissues and/or sorted prior to analysis. Therefore, analyses at the mRNA and protein level must be conducted at very low levels and substantial engineering opportunities exist in the biological field to provide the necessary solutions. However, generation of the data is only the first hurdle, as the analysis of the data from a systems perspective then must be undertaken. Hood presented systems biology as the challenge for the 21st century and provided a series of examples of large-scale approaches to biology, from genome sequencing of unicellular organisms, to the sequencing of the T-cell receptor locus, to cancer biology, all of which benefit from such approaches.

Three other presentations were included in the program, to provide a broader background to the utilization of proteomics in drug discovery. Doug Buckley (Exelixis) described the generic view of the drug discovery pipeline, the various "choke" points in the process, and where proteomics could play a role. Of note was the discussion of the changing patent protection landscape, during which Buckley said that full-length cDNA patents were being issued despite the existence of EST patents on portions of these genes. Buckley also predicted that functional data is expected to be required for patents beyond the inferences gained from bioinformatics. The choke points he referred to were target validation, assay development, mechanistic biology, and toxicology. Exelixis is using model organisms (*Caenorhabditis elegans*, *Drosophila*, mouse, and zebrafish) to screen for genes that disrupt/modulate pathways common between man and these organisms. Roles for proteomics included follow-up on targets (direct analysis of protein differences, proteins associated with gene products of interest), assay development [e.g., validation of hits in high-throughput screening (HTS)], and mechanistic biology (e.g. comprehensive analysis of a knockout phenotype). Most importantly, Buckley presented the bottom line that all new technologies must demonstrate their worth by concrete changes in the drug development pipeline (i.e., greater efficiency, better decisions). He predicted that proteomics could provide these benefits at the multiple restriction points referred to above.

Pharmacoproteomics, using 2-DE to profile mechanisms of drug efficacy and toxicity, was presented by Tina Gatlin (Biosource/Large Scale Biology Corporation). The synergy between mRNA expression profiling (for low-abundance gene products) and protein expression profiling (for posttranslational modifications and subcellular localization) was presented. An exception to this is the search for surrogate markers, where secreted proteins were normally the choice and in which there is no identifiable mRNA source to mirror serum or urine protein expression. The aim of their Molecular Effects Database of 2-DE patterns, obtained from livers of drug-treated rats, is to establish links between expression patterns and toxic endpoints to reveal markers for efficacy and prediction of side effects which can be used for lead selection. In disease models, the hypothesis is that the altered expression pattern could be reversed by treatment with a drug.

The closing presentation of the meeting, given by Jeff Seilhamer (Incyte), presented analyses of the precursor to proteins, mRNA. The staff at Incyte have generated very large EST libraries and from these have estimated the number of genes in the human genome to be 129,769 (based on CpG island estimates, 142,634). They are now sequencing the human genome at a rate of about 1 million reads a month on the Megabace platform with 9 sequencing runs/day. Assembly of the data is being accomplished using Linux on 1,500 CPUs (160 computers) with 75 terabytes of storage capacity. Single-nucleotide polymorphisms (SNPs) are being calculated from their sizable EST collection, and mRNA expression profiling is being achieved using their GEM microarray platform. These data are being integrated with 2-DE proteomics data being generated by their partner Oxford GlycoSciences. This integration of the technologies of genomics and proteomics forms the basis of their drug discovery approach for profiling differences between normal and diseased tissue.

Computational aspects of proteomics. Determining the masses of peptides (MS spectra) derived from enzymatic digestion of gel-separated proteins is often the first step in a mass spectrometric-based protein identification strategy. Peptide-mass mapping is the most commonly employed mass spectrometric approach for protein identification from organisms whose genome is completely sequenced (or at least for which the more abundantly expressed genes have been sequenced). The basis of the method is the matching of experimentally determined peptide masses with peptide masses calculated for each entry in a sequence database (using the specificity of the enzyme used to generate the experimental data). How well the experimentally determined masses match with the calculated masses forms the basis of the approach. Ron Beavis (Proteometrics) described how to obtain high-quality data, which even if less, are better than more low-quality data. The use of specific matrices as well as the use of standards with respect to obtaining appropriate data sets for peptide-mass mapping was addressed. Later in the day David Fenyo (Proteometrics) described how to utilize this data in a three-step process as is performed in their WWW-available program, Profound, which uses a Bayesian algorithm (**http://www.proteometrics.com**). The process is as follows:

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1) assignment of monoisotopic masses to the raw data, *2*) peptide-mass search, and *3*) significance testing of the result (4). The last step was presented as the most critical because it is from this that the confidence of the match is derived. This is achieved through calculation of a score frequency function for false positives. This was derived from statistical analysis of the database being searched using random selections of peptide masses from different proteins that are then grouped as synthetic proteins and used in a peptide-mass search of the database in question. This is repeated for a variety of random selections to come up with robust statistics for false positives.

The next level of protein identification is the generation of fragment ion spectra from peptides isolated in the gas phase of the mass spectrometer (MS/MS spectra). Matching of fragment ion spectra follows the same principle as for peptide-mass mapping. Experimentally calculated masses of fragment ions (together with the intact mass of the peptide, and often the specificity of the enzyme used to generate the peptide) are matched with those calculated for isobaric peptides (i.e., same mass as experimentally determined) from entries in sequence databases. Arthur Moseley (Glaxo Wellcome) described how nanoscale capillary LC-MS/MS (where peptides are separated chromatographically before MS/MS) had been automated for identification of gel-separated proteins. The throughput of this 75 μm ID capillary system connected to a Qq-TOF mass spectrometer was 20 samples per day at levels to 30 fmol (loaded on gel) for BSA. Moseley continues to develop ultra-HPLC (in some cases combined with variable flow systems) that improve both the speed and resolution of separation. In a Glaxo Organelle Proteomics program, various approaches to protein identification were examined. A comparison of the total number of proteins identified following in situ enzymatic digestion of proteins separated by either high-resolution 2-DE or one-dimensional (usually SDS-PAGE) gel electrophoresis (1-DE) was presented. Only one or a limited number of proteins are present in each of the 2-DE spots, whereas many proteins were present in the 1-DE bands of the enriched Golgi complex. In fact, more proteins were identified from the 1-DE bands than from the 2-DE spots (see below, *Analysis of complex protein mixtures without gel electrophoresis*).

MS/MS spectra derived from tryptic digestions conducted in the presence of equal quantities of $H_2{}^{16}O$ and $H_2{}^{18}O$, when combined with subtractive analysis of the two types of spectra, allows de novo sequencing as described by Matthias Wilm (EMBL) (18). By utilizing a Qq-TOF mass spectrometer, peptides containing both COOH-terminally incorporated stable isotopes and just the isoform containing the ^{18}O could be selected for fragmentation from the mixture. Subtraction of the ^{18}O spectrum from the ^{16}O:^{18}O spectrum reveals only ^{16}O *y*-series ions. Often, a complete ion series is obtained. The method has proved feasible in their hands when 1 pmol of protein is present in the gel (1/4 of this amount can be successfully analyzed with standard digest conditions).

Automated identification of gel-separated proteins by mass spectrometry. Following quantitative analysis of 2-DE patterns, the next step is the identification of all protein spots that display differential expression. Andrew Gooley (Proteome Systems) described the approaches they are employing for quantitative analysis using 2-DE. This included the following: sample preparation (sequential detergent extraction with aminosulfobetaine-14), narrow-range immobilized pH gradient (IPG) with mini-gels for the 2nd dimension, through to the robotic system that they have codeveloped for spot excision, liquid handling (peptide extraction and reverse-phase bead cleanup and storage) and peptide-mass fingerprinting by MALDI-MS. Apart from the throughput of the robotic system, diminished contamination from keratin and more reproducible spotting of samples for MALDI-MS is a highly desirable feature of automation. Hans-Werner Lahm (Hoffmann-La Roche) described the high-throughput system they use for automated spot excision from 2-DE, digestion (with low-salt buffer to eliminate the need for cleanup), and spotting for automated MALDI-MS. Lahm also described the computational aspects of operating such a system in high-throughput mode for long periods of time, including automated database search routines for users distributed throughout the world at other Roche sites. They are investigating the use of stable isotope labeling ($^{14}N/^{15}N$) followed by mixing of each sample prior to 2-DE for direct quantitation of relative expression differences from the MALDI-MS spectra of individual protein spots. The system averages 1,000 spots to spectra per day (including downtime).

David Arnott (Genentech) described automation of in-gel digestions following analysis of differentially regulated proteins from 2-DE. Arnott described the trapping cartridge approach that was required to analyze extracted peptides from the DigestPro robot (currently 30 sample spots, but upgradeable to 96) by microcapillary LC-MS/MS. They aimed to automate as much of the sample processing as possible with automated liquid handling from the digestion robot to the data-dependent LC-MS/MS (capable of handling 40 samples per day) using an ion-trap mass spectrometer followed by auto-database searching using Sequest (3). The system is capable of analysis of subpicomolar quantities of protein from silver-stained gels.

Advances in separations and mass spectrometers. Accurate mass analysis of intact proteins using an 11.5-T FT-ICR coupled with a capillary electrophoresis (CE) instrument was demonstrated by Richard Smith (Pacific Northwest National Laboratory) as a means of proteome analysis. Through the use of stable isotope labeling of one sample and running that sample with an unlabeled sample provides the possibility to measure protein expression ratios. To identify the proteins that display different ratios, dissociation in the FT-ICR-MS to yield mass tags is possible. Having intact mass information as well as identification allows post-translational modifications to begin to be investigated. The mass accuracy obtainable by this FT-ICR-MS was said to be <0.75 ppm which allows the generation of

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accurate mass tags for tryptic peptides. In many cases this may be sufficient for protein identification (at least for an organism like *C. elegans*). In some cases, MS/MS may be required, but once performed it would not have to be repeated. Another possibility is the identification of cysteine-containing peptides at a mass accuracy of 1 ppm, which was said to be sufficient for identification. Another possibility being explored with this instrument is multiplexed MS/MS, where up to 7 ions could be isolated at once and the MS/MS spectrum could be deconvoluted for each selected ion (requires accuracy of <10 ppm). This will be tried with online separations in the near future.

Marvin Vestal (PE BioSystems) described his continuing efforts in MALDI-MS instrument design. The attributes he is aiming for include sensitivity, specificity (resolution, mass accuracy, selective ionization), speed, accuracy, dynamic range, and mass range. The sensitivity will always be limited by chemical noise, but the aim is to reduce the limitations of ionization and data handling. Vestal would like to achieve a sensitivity of 1 fmol with a data acquisition rate of 1 spectrum/s. The instrument he is designing to achieve these aims is a MALDI-TOF/TOF-MS. This system has an ion gate (with 500 resolution and no loss of sensitivity) after the collision cell so that metastable ions created after reacceleration are removed. Although this system is in the early stages of development, the data shown demonstrate that this instrument is meeting most of the stated objectives.

The hybrid quadrupole TOF (Qq-TOF) mass spectrometer developed a few years ago (9), which has now been commercialized, utilizes an ESI for ionization (10). Both Ken Standing (Univ. of Manitoba) and Brian Chait (Rockefeller Univ.) described the use of a MALDI ion source for introduction of ions into a modified commercial Qq-TOF, thus taking advantage of both the high-efficiency ion production of the MALDI and the ion isolation/fragmentation of the quadrupole system with a TOF mass analyzer. Standing presented data showing sensitivity of purified standards (e.g., Substance P) in the 70 amol range (1-min acquisition) for MS and 7 fmol for MS/MS with 10,000 resolution. This instrument offers similar advantages to the TOF/TOF described above.

Online MS analysis of capillary electrophoretic or chromatographic separations of peptide (or proteins) is most often achieved using ESI-MS. Barry Karger (Barnett Institute) described how very small quantities of peptides/proteins could be separated and analyzed using vacuum deposition onto Mylar audio tape for subsequent coupled MALDI-MS analysis. The approach had so far been multiplexed with the effluent of 12 capillaries being deposited under vacuum onto the tape. The approach is designed for high-throughput separations and mass analysis.

Proteomic analyses often employ 2-DE, but David Lubman (Univ. of Michigan) described a liquid-phase 2-D separation of proteins utilizing a novel MS. The requirements of his mass spectrometer were high sensitivity, low duty cycle, and fast response. He designed and built an ion trap to capture ions from the CE coupled to TOF-MS. The 2-D liquid-phase separation consisted of nonporous silica bead RP-HPLC (which provided good resolution <50 kDa) that was conducted at high pH followed by CE and MS. Whole cell lysates were analyzed with this system, and some of the data obtained were presented.

Biological applications. Brian Chait (Rockefeller Univ.) presented the culmination of an enormous amount of work at both the protein chemistry (mass spectrometry) and cell biology levels. The nuclear pore complex (NPC) in yeast is a massive structure (1,000 Å across with 8-fold symmetry) that regulates protein transport in/out of the nucleus. The first step in understanding this structure was to purify the complex and then identify every protein present. The protein fraction was separated by several different chromatographic steps followed by SDS-PAGE from which every visible band was excised and analyzed by MALDI-IT-MS. This was an especially daunting task as the NPC when isolated contains a snapshot of the proteins transiting the NPC at that point in time. Hence, of the 174 proteins identified, 29 were nucleoporins and only 14 were shown to be present in the NPC. These 14 proteins were characterized as being present in the NPC by a variety of techniques. Protein A (4.5 repeats of the Fc binding region) fusions with the proteins of interest were generated, and immunohistochemistry was performed on cells transfected with these constructs. Electron microscopy of hundreds of NPCs following transfection allowed stoichiometry and symmetry (nuclear/cytoplasmic/asymmetric) to be determined. Subcellular fractionation and high-pH extractions were also performed to further characterize localization biochemically. This elegant study has allowed a testable model for nuclear transport to be constructed.

Two examples of the utility of analysis of unfractionated or partially fractionated complex protein mixture digests (see next section) were presented by Scott Patterson (Amgen Inc.). As a first step in the understanding of the interchromatin granule clusters (IGC), a nuclear organelle which is a major site of mRNA splicing. Samples enriched in this structure were digested with trypsin, and the complex mixture of peptides was analyzed by data-dependent LC-MS/MS (8). Some proteins known to be present in these structures were identified together with 19 novel genes (including ESTs): Three of the genes were confirmed to be present in the IGC by immunohistochemistry of cells transfected with yellow fluorescent protein (YFP)-fusion constructs with counter staining of splicing factors. The other study presented identified 108 proteins present in a protein fraction obtained from isolated mitochondria treated with atractyloside [mimicking in vitro the permeability transition pore complex (PTPC) which occurs during apoptosis] (13).

Analysis of immunoprecipitates using a new affinity strategy was presented by Gitte Neubauer (EMBL). The new strategy is referred to as tandem affinity purification (TAP) and was developed by colleagues at EMBL (15). The system utilizes a double tag for higher

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specificity and much reduced background. The human spliceosome immunoprecipitated under normal conditions (see Ref. 5 for same approach with yeast tri-snRNP) and using the TAP method were compared, demonstrating the utility of this approach.

The common theme of all of these applications is that MS was utilized early on to provide rapid and accurate protein identifications. The genes identified could then be further analyzed to attempt to determine their function.

The use of MS to identify proteins from 2-DE gels was also described by Al Burlingame (Univ. of California, San Francisco) and Reid Townsend (Oxford GlycoSciences). Burlingame described their work to identify protein targets of acetaminophen during acute toxicity and the intricacies of such analyses (14). Townsend described an Oxford GlycoSciences and Pfizer collaboration to separate by 2-DE and identify proteins from cerebrospinal fluid (CSF) in a study aimed at identifying markers for Alzheimer's disease. CSF is a compartment isolated by the blood-brain barrier but it is not just a filtrate of blood. It is produced by the choroid plexus and has a total central nervous system volume of about 90–150 ml that is turned over a few times per day. Comparative analysis of matched plasma CSF samples (in addition the normal/diseased samples) revealed that key plasma proteins (e.g., albumin, transferrin, IgG) showed markedly different relative ratios between plasma and CSF. For effective 2-DE analysis of these samples, a selective removal of albumin, IgG, transferrin, and haptoglobin had to be developed. This was accomplished by affinity depletion. Interestingly, many features in a 2-DE separation are albumin fragments (in fact, 4% of total features). Their study included 512 samples from 228 patients and resulted in 1,131 features (spots) being annotated. Potential markers of Alzheimer's disease were said to be identified.

Separate from the MS identification issues covered in most of the meeting, Kerstin Strupat (Univ. of Muenster) presented her work on MS analysis of noncovalent complexes. Here the challenge is to transfer noncovalent interactions that occur in the condensed phase to the gas phase. ESI-MS has been shown by a number of groups to work, but MALDI-MS analysis has proved more difficult. Examples of MALDI-MS analysis of noncovalent protein:protein (streptavidin tetramer and the macrophage migration inhibitory factor related proteins MRP-8 and MRP-14) and protein:ligand (aldose reductase:NADP) interactions were presented. Interestingly, analysis of the first laser pulse during a MALDI-MS analysis often allows investigation of noncovalent interactions that are not observed during subsequent pulses (16).

Analysis of complex protein mixtures without gel electrophoresis. The first stage of many proteome projects is the identification of the components comprising the system under study. This is of course the first step in understanding any biological system. As described above, an increasing (but still limited) number of laboratories have access to robotic systems requisite for the analysis of large numbers of spots from 2-DE. However, a trend in the field is emerging toward the elimination of the high-resolution protein separation step prior to protein identification by MS. In this approach, the entire enriched protein fraction is enzymatically digested (usually with trypsin), and the resulting complex peptide mixture is subjected to data-dependent LC-MS/MS. In this approach the peptides are separated by both hydrophobicity (RP-HPLC) and charge (m/z in the mass spectrometer) prior to ion selection by the MS control software (hence, data dependent). At this meeting, presentations from five groups demonstrated the utility of the approach to identify components of complex mixtures.

Analysis of immunoprecipitated proteins or enriched protein fractions (e.g., Golgi complex) by either gel electrophoresis followed by in-gel digestion and MS or digestion of the entire protein fraction and analysis by data-dependent LC-MS/MS using a Qq-TOF was described by Jyoyti Choudhary (Glaxo Wellcome). Batched MS/MS spectra were searched using the Mascot program (**http://www.matrixscience.com**). Data presented showed that if the immunoprecipitate was clean, then direct digestion of the mixture proved slightly more successful than analysis of gel-separated proteins. When an enriched Golgi complex from rat liver was separated by either 2-DE (135 spots) or 1-DE (77 bands) and in-gel digested followed by LC-MS/MS, significantly more proteins were identified from the 1-DE separation.

David Arnott (Genentech) described the proteomics component of Genentech's Secreted Protein Discovery Initiative, which also includes genomic, signal trap, expression, and functional analysis. Arnott evaluated three methods to identify proteins secreted from human umbilical microvascular endothelial cells (HUMECs) into 60 ml of serum-free media; 2-DE and 1-DE (with/without staining) followed by in-gel digestion, and direct digestion of the entire protein mixture. Digests were analyzed using the microcapillary system described above. Interestingly, direct digestion followed by data-dependent LC-MS/MS identified the most proteins, but all three methods were complementary in their hands (21 proteins identified by all three methods but no completely novel gene products).

Analysis of serum fractionated using the Cohn pH/ethanol precipitation protocol followed by digestion of the entire fraction prior to data-dependent LC-MS/MS was described by Karl Clauser (Millennium Pharmaceuticals) in the context of the studies of differences between wild type and ApoE −/− mice. Clauser also presented the bioinformatics flow for data handling, which utilizes a variant of the publicly available MS-Tag (**http://prospector.ucsf.edu/**) for protein identification and a de novo sequence interpretation program referred to as SHERENGA (2). Their stated aim is for searching to keep up with the LC-MS/MS. They have also been experimenting with the IEX ion-exchange protocol developed by Andy Link (7) as a means of decreasing the complexity of the sample and reducing the number of singly charged and highly charged ions as these are less likely to be identified. In one IEX

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fraction, 87 plasma proteins were identified in a single run compared with 66 from an unfractionated sample.

Scott Patterson (Amgen) described Amgen's proteomics efforts, now in the third year. They are employing data-dependent LC-MS/MS of complex protein mixture digests. The stated aim is to reduce the complexity such that in an ideal situation only one peptide for each protein in the mixture is fragmented during LC-MS/MS. To achieve this aim, various affinity methods can be employed, and the use of cysteinyl peptide capture using either thiopropyl Sepharose or a biotin alkylating reagent, *N*-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio)-propionamide (HPDP-biotin), was described (13). The former was used in a large-scale analysis of urinary proteins where digestions of the unfractionated starting material, albumin/IgG depleted, or cysteinyl peptide captured or noncaptured were analyzed. The samples were analyzed with replicate LC-MS/MS runs using narrow mass ranges for ion selection for each run, thereby increasing the number of unique spectra selected for fragmentation. This analysis resulted in the identification of over 200 proteins, including a number of uncharacterized nucleotide sequences (e.g., ESTs). Smaller scale analyses are described above, in one case [soluble intermembrane proteins (SIMPs)] utilizing cysteinyl peptide capture to identify more proteins than with no fractionation. Data handling for this high-throughput effort was also briefly described. A number of the fractions being analyzed have some of the same components; therefore, to enhance the identification process, spectral matching of the database (>5 million spectra) is performed. This links identical spectra and therefore reduces the redundancy associated with re-searching already identified spectra.

Quantitative analysis of two samples without electrophoresis. MALDI-MS, using the surface enhanced laser desorption ionization (SELDI) surface, to search for disease markers in biological fluids was presented by Scot Weinbeger (Ciphergen Biosystems). In this approach, defined chemical/biochemical surfaces are utilized to allow fractionation of proteins from biological fluids in a reproducible manner. This reproducibility allows comparisons between different samples to be made. Weinberger described the search for markers of benign prostatic hyperplasia that, like prostate cancer, displays elevated prostate specific antigen (PSA) levels. The fraction exhibiting a difference between these samples was able to be enzymatically digested, and a number of peptides were generated. These were able to be fragmented using the MALDI Qq-TOF of Standing, described above. It appears as though there is a difference in the relative level of a seminogelin fragment between these two diseases, providing a potential differential marker. The method is sensitive but apparently limited to analysis of proteins less than about 20 kDa (a range not well characterized by 2-DE).

A combination gel/MS approach referred to as a "virtual 2-D gel" was presented by Phil Andrews (Univ. of Michigan). In this approach, proteins are separated by charge using thin-layer isoelectric focusing (IEF), and this gel is then subjected to MALDI-MS. By rastering through the entire IEF gel, a composite display of all acquired MALDI-MS spectra can be generated (hence, the virtual 2-DE). Such analyses would provide very accurate mass measurements, greatly assisting in posttranslational modification analyses as well as potentially quantitation.

Karl Clauser (Millennium Pharmaceuticals) described their efforts at utilizing already existing LC-MS/MS data to attempt to gain some quantitative/qualitative information as to differences between samples. Differences in serum protein levels between wild-type and ApoE −/− mice have been examined using this approach, which compares the MS ion current from peptides identified between LC-MS/MS runs of each sample. Comparison between runs is a difficult task, but data suggested that there is sufficient confidence to state a significant difference if there is a difference of a factor of 3 between some components of the samples.

An LC-MS/MS-based system was described by Steve Gygi (Univ. of Washington) for quantitative analysis of complex mixtures. The technology is referred to as isotope-coded affinity tag (ICAT) (6). The ICAT reagent described here is composed of three units: an affinity reagent (biotin), a linker region (one of two forms), and a reactive group (a thiol-specific reagent, iodoacetic acid). The linker region encodes the mass difference, with the light version having 8 hydrogens and the heavy version having 8 deuteriums. Thus the mass difference is 8 mass units (doubly charged ions will have an *m/z* difference of 4). Following reduction and alkylation of each of the two protein samples with one of the two reagents, the two samples can then be mixed together. All subsequent manipulations are performed as a mixture, culminating in tryptic digestion of the complex sample and capture of the cysteinyl peptides on avidin. The bound peptides are released and analyzed by LC-MS/MS, revealing paired signals of peptides. Calculation of areas under the peak for each paired ion from the LC-MS data provides an accurate record of the relative quantities of the proteins from each starting sample. The MS/MS spectra allow identification of the peptides. The approach was elegantly demonstrated with yeast grown on either galactose-containing media or ethanol-containing media. Proteins expected to be differentially regulated were observed, and, highlighting the advantages of analysis at the protein level as opposed to the mRNA level (e.g., microarray), alcohol dehydrogenase-1 (ADH1) was found to be oppositely regulated (as expected) to ADH2, to which it is 95% homologous. This is a very promising approach for quantitative analysis of complex protein mixtures.

A number of interesting posters were also presented at the meeting, and some of the presenters were given the opportunity to "advertise" their posters. These dealt with the same range of subjects presented in the oral sessions.

Conclusion. The organizers Ruedi Aebersold and John Stults brought together an excellent program for this meeting, with essentially all major laboratories in

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this field being represented. The field has grown enormously over the past few years, and advancements presented at this meeting indicate an optimistic view of the future for proteomics. This very successful meeting provided the 162 attendees with the state-of-the-art in mass spectrometry and proteomics.

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REFERENCES

1. **Carr SA and Annan RS.** Overview of peptide and protein analysis by mass spectrometry. In: *Current Protocols in Molecular Biology*, edited by Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, and Struhl K. New York: Wiley, 1997. p. 10.21.1–10.21.27.
2. **Dancik V, Addona TA, Clauser KR, Vath JE, and Pevzner PA.** De novo peptide sequencing via tandem mass spectrometry. *J Comput Biol* 6: 327–342, 1999.
3. **Eng JK, McCormack AL, and Yates JR, III.** An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database. *J Am Soc Mass Spectrom* 5: 976–989, 1994.
4. **Eriksson J, Chait BT, and Fenyo D.** A statistical basis for testing the significance of mass spectrometric protein identification results. *Anal Chem* In press.
5. **Gottschalk A, Neubauer G, Banroques J, Mann M, Luhrmann R, and Fabrizio P.** Identification by mass spectrometry and functional analysis of novel proteins of the yeast [U4/U6.U5] tri-snRNP. *EMBO J* 18: 4535–4548, 1999.
6. **Gygi SP, Rist B, Gerber SA, Turecek F, Gelb MH, and Aebersold R.** Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nat Biotechnol* 17: 994–999, 1999.
7. **Link AJ, Eng J, Schieltz DM, Carmack E, Mize GJ, Morris DR, Garvik BM, and Yates JR, III.** Direct analysis of protein complexes using mass spectrometry. *Nat Biotechnol* 17: 676–682, 1999.
8. **Mintz PJ, Patterson SD, Neuwald AF, Spahr CS, and Spector DL.** Purification and biochemical characterization of interchromatin granule clusters. *EMBO J* 18: 4308–4320, 1999.
9. **Morris HR, Paxton T, Langhorne J, Berg M, Bordoli RS, Hoyes J, and Bateman RH.** High sensitivity collisionally-activated decomposition tandem mass flight mass spectrometer. *Rapid Commun Mass Spectrom* 10: 889–896, 1996.
10. **Morris HR, Paxton T, Panico M, McDowell R, and Dell-A.** A novel geometry mass spectrometer, the Q-TOF, for low femtomole/attomole-range biopolymer sequencing. *J Protein Chem* 16: 469–479, 1997.
11. **Patterson SD.** Protein identification and characterization by mass spectrometry. In: *Current Protocols in Molecular Biology*, edited by Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, and Struhl K. New York: Wiley, 1998. p. 10.22.1–10.22.24.
12. **Patterson SD and Aebersold R.** Mass spectrometric approaches for the identification of gel-separated proteins. *Electrophoresis* 16: 1791–1814, 1995.
13. **Patterson SD, Spahr CS, Daugas E, Susin SA, Irinopoulou T, Koehler C, and Kroemer G.** Mass spectrometric identification of proteins released from mitochondria undergoing permeability transition. *Cell Death Differ* In press.
14. **Qiu YC, Benet LZ, and Burlingame AL.** Identification of the hepatic protein targets of reactive metabolites of acetaminophen in vivo in mice using two-dimensional gel electrophoresis and mass spectrometry. *J Biol Chem* 273: 17940–17953, 1998.
15. **Rigaut G, Shevchenko A, Rutz B, Wilm M, Mann M, and Seraphin B.** A generic protein purification method for protein complex characterization and proteome exploration. *Nat Biotechnol* 17: 1030–1032, 1999.
16. **Vogl T, Roth J, Sorg C, Hillenkamp F, and Strupat K.** Calcium-induced noncovalently linked tetramers of MRP8 and MRP14 detected by ultraviolet matrix-assisted laser desorption/ionization mass spectrometry. *J Am Soc Mass Spectrom* 10: 1124–1130, 1999.
17. **Wilkins MR, Sanchez J-C, Gooley AA, Appel RD, Humphery-Smith I, Hochstrasser DF, and Williams KL.** Progress with proteome projects: why all proteins expressed by a genome should be identified and how to do it. *Biotechnol Genet Eng Rev* 13: 19–50, 1995.
18. **Wilm M, Neubauer G, and Bachi A.** Automatic de novo sequencing of proteins by differential scanning with a quadrupole time of flight instrument. *Proc 47th ASMS Conf Mass Spectrom Allied Topics Dallas June 13–17 1999*, p. 1541.
19. **Wilm MS and Mann M.** Electrospray and Taylor-Cone theory, Dole's beam of macromolecules at last? *Int J Mass Spectrom Ion Processes* 136: 167–180, 1994.

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Mass spectrometry and proteomics

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V. Thongboonkerd, E. Gozal, L. R. Sachleben Jr., J. M. Arthur, W. M. Pierce, J. Cai, J. Chao, M. Bader, J. B. Pesquero, D. Gozal and J. B. Klein
J. Biol. Chem., September 20, 2002; 277 (38): 34708-34716.
[Abstract] [Full Text] [PDF]

New Concepts in Hypertrophic Cardiomyopathies, Part II
R. Roberts and U. Sigwart
Circulation, October 30, 2001; 104 (18): 2249-2252.
[Full Text] [PDF]

Neural Model of the Genetic Network
J. Vohradsky
J. Biol. Chem., September 28, 2001; 276 (39): 36168-36173.
[Abstract] [Full Text] [PDF]

Proteome Analysis of Metabolically Engineered Escherichia coli Producing Poly(3-Hydroxybutyrate)
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J. Bacteriol., January 1, 2001; 183 (1): 301-308.
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